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Original Article

Altered intestinal bile salt biotransformation in a cystic fibrosis (*Cftr*$^{-/-}$) mouse model with hepato-biliary pathology

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Abstract

Background: *Cftr*$^{-/-tm1Unc}$ mice develop progressive hepato-biliary pathology. We hypothesize that this liver pathology is related to alterations in biliary bile hydrophobicity and bile salt metabolism in *Cftr*$^{-/-tm1Unc}$ mice.
Methods: We determined bile production, biliary and fecal bile salt- and lipid compositions and fecal bacterial composition of C57BL/6 J *Cftr*$^{-/-tm1Unc}$ and control mice.
Results: We found no differences between the total biliary bile salt or lipid concentrations of Cftr$^{-/-}$ and controls. Compared to controls, Cftr$^{-/-}$ mice had a ~30% higher bile production and a low bile hydrophobicity, related to a ~7 fold higher concentration of the choleretic and hydrophilic bile salt ursocholate. These findings coexisted with a significantly smaller quantity of fecal Bacteroides bacteria.
Conclusions: Liver pathology in *Cftr*$^{-/-tm1Unc}$ is not related to increased bile hydrophobicity. *Cftr*$^{-/-}$ mice do however display a biliary phenotype characterized by increased bile production and decreased biliary hydrophobicity. Our findings suggest Cftr dependent, alterations in intestinal bacterial biotransformation of bile salts.

Keywords: Cystic fibrosis; Liver disease; Bile salts; Ursocholic acid; Mice model; Intestinal bacterial microflora; CFTR

1. Background

The pathogenesis of cystic fibrosis related liver disease (CFLD) is not known (1,2). Most information, on CFLD in humans, has been derived from post-mortem evaluations (3). Liver histology shows a biliary cirrhosis with bile duct proliferation and damage pointing in the direction of a biliary origin of the disease (1,3). Prospective histological studies in humans are scarce, mainly because of the need for invasive diagnostic procedures in asymptomatic patients (4).

Animal models can, theoretically, help to explain how liver disease in CF develops. Most CF mouse models do not display any liver pathology (5). However, the C57BL/6 J *Cftr*$^{-/-tm1Unc}$ mice offers the opportunity to study liver disease, since it does show, progressive, CF like liver pathology, with increasing age, that is not present control mice (6). Additionally, Freudenberg et al. reported that older ΔF508 CF mice (background 75% C57BL/6, 25% 129SvEv) also had more liver fibrosis than their wild-type controls (7). The fact that these ΔF508 CF mice only develop a liver phenotype at a very high age (100–200 days) makes them less suitable to study CFLD development.

Biliary bile salt hydrophobicity and cytotoxicity are frequently involved in the development of cholestatic liver diseases (8).

Abbreviations: CF, Cystic fibrosis; Cftr, Cystic fibrosis transmembrane conductance regulator; CFLD, Cystic fibrosis related liver disease; Unc, University of North Carolina; CA, Cholic acid; DCA, Deoxycholic acid; CDCA, Chenodeoxycholic acid; HDCA, Hyodeoxycholic acid; UDCA, Ursodeoxycholic acid; UCA, Ursocholic acid; MCA, Muricholic acid.

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Primary bile salts are synthesized in the liver and are secreted, via the bile, into the intestine. In the gut, primary bile salts are transformed to secondary bile salts by the intestinal microflora. Differences in intestinal bacterial flora composition induce variations in bile salt composition. After passage through the gut, most bile salts are absorbed in the terminal ileum and return to the liver as part of the enterohepatic circulation (9).

As a result of the intestinal biotransformation, a variety of bile salts is formed. Individual bile salts have variable physiological properties. Hydrophobic bile acids are more cytotoxic than hydrophilic bile salts. On the other hand, when secreted into bile, hydrophilic bile salts are more choleretic than hydrophobic bile salts. The cytotoxicity of biliary bile salts is determined by their concentration, their composition and the presence of additional biliary lipids like phospholipids.

We hypothesized that an increased biliary bile salt hydrophobicity (and thus, cytotoxicity) plays a role in the early genesis of the liver pathology of the *Cftr*$^{-/-tm1Unc}$ mice. To address this hypothesis, we characterized bile production and biliary and fecal bile salt excretion and composition in young CF mice and controls.

2. Methods

2.1. Animals

All experimental protocols were conducted in the Hospital for Sick children, the Research Institute, Toronto, Canada, after approval by the institutional Animal Care Committee. Mice were bred to wild-type C57BL/6 J mice, obtained from the Jackson Laboratories (Bar Harbor, ME). To minimize bowel obstruction and optimize long-term viability, 20- to 23-day-old congenic C57BL/6 J *Cftr*$^{-/-tm1Unc}$ mice and their *Cftr*$^{+/+}$ littermates were weaned to a liquid diet (Peptamen, Nestlé Nutrition, Canada). Mice were housed in a non-sterile conventional housing unit in micro-isolators cages, with corncob bedding changed daily, and provided with sterile water in addition to the liquid diet. The colony was maintained at a pathogen-free status by serological screening at a commercial laboratory. The animals used for the experiments were approximately 1.5–4 months old.

2.2. Experimental procedures

All experiments were performed in mice from the same colony. We used *Cftr*$^{-/-}$ mice (N = 9, four males) and control mice (N = 9, three males). At the start of the experiment, mice were housed in metabolic cages. The total fecal production of individual mice was collected for three consecutive days, dried and homogenized. After overnight fasting, bile was collected after prior surgical ligation of the common bile duct and gall bladder cannulation using silicone tubing (size 0.020″ × 0.037″, Degania Silicone Ltd.) under anesthesia. The anesthetic mixture consisted of: 0.75 ml ketamine (100 mg/ ml), 0.25 ml xylazine (20 mg/ ml) and 4 ml saline. The mixture (0.1 ml per 10 gram body weight) was intraperitoneally administered. Body temperature was maintained by placement of the animal in a temperature and humidity controlled incubator. After a 10 minutes equilibration period, bile secretions were collected in 3 periods of 30 minutes. Bile flow rate was assessed gravimetrically, assuming that 1 g of secretion corresponds to 1 ml. The mice were euthanized after the bile collection, and the liver was harvested.

2.3. Liver histology

Hematoxylin and eosin (H&E) slices of the whole liver were assessed in a blinded fashion by an expert hepato-pathologist (JP) for the degree of portal inflammation, bile duct changes and bridging fibrosis. We applied a previously published numerical scoring system for each parameter, using an arbitrary 0 to 4 scale, in which zero represents normal histology, and four represents the most severe pathology for each parameter (6). A minimum of five portal tracts was assessed for each mouse, and an average of the numerically scored parameters provided a total overall score for each animal.

2.4. Analytical techniques

Biliary BS concentrations in bile and feces were determined by an enzymatic fluorimetric assay (10). Lipids were extracted from the bile (11). The phospholipid and cholesterol concentrations were determined using a spectrophotometric assay (12). Biliary BS composition in bile collected in first 30 minute period) and in feces were determined by extracting the bile salts with commercially available Sep-Pak-C18 (Mallinckrodt Baker, Deventer, The Netherlands) cartridges and converting them to their methyl ester/trimethylsilyl derivatives (13). Bile salt profiles were analyzed using capillary gas chromatography. The hydrophobicity of BS in bile was calculated according to the Heuman index based on the fractional contribution of the major BS species (14).

2.5. Bacterial DNA

Bacterial DNA was isolated from 150 mg of dried homogenized feces by commercially available QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). Concentration of bacterial DNA was measured by spectrophotometry (NanoDrop 2000c, Thermo Scientific, Wilmington, DE) and 50 ng (15 ng respectively for universal primer) of DNA was used for amplification by RT-PCR with SensiMix™ SYBR® Hi-ROX Kit (Bioline, Taunton, MA) using bacterial groups specific primers for 16S (supplementary materials: Table 1).

2.6. Statistical analysis

Analyses were performed using IBM SPSS Statistics version 22 (IBM corp., United states). All quantitative results are reported as means ± SEM and ordinal results as means with range. Differences between study groups were evaluated using the Mann-Whitney U test. Bile flow was additionally analyzed by correlation and regression analysis. The level of significance was set at a P value of less than 0.05.

3. Results

3.1. Histology

The mean age of the *Cftr*$^{-/-}$ and control mice was similar (75 ± 25 days). We found a significantly higher presence of bile duct changes (*Cftr*$^{-/-}$: 0.7 (range: 0–2) vs. *Cftr*$^{+/+}$: 0.1 (range: 0–1); respectively; $P < 0.05$) and bridging fibrosis (*Cftr*$^{-/-}$: 1.1 (range: 0–4) vs. *Cftr*$^{+/+}$: 0; respectively; $P < 0.05$) in *Cftr*$^{-/-}$ mice compare to controls. However, histology did not differ between *Cftr*$^{-/-}$ mice and controls with respect to portal inflammation (*Cftr*$^{-/-}$: 1.5 (range: 0–4) vs. *Cftr*$^{+/+}$: 0.5 (range: 0–2); respectively; NS). Our current histological findings were, to a large extent, consistent with the previously reported liver pathology in this mouse model (6).

3.2. Bile production and bile salt secretion

We found that the bile production was 20–30% higher in *Cftr*$^{-/-}$ mice compared to controls ($P < 0.05$ in the last sample period, Fig. 1A). The total biliary bile salt concentration and total biliary bile salt secretion rate were not different between *Cftr*$^{-/-}$ mice and controls (Fig. 1B and 1C). Bile salt-dependent flow and bile salt independent flow were derived. Regression analysis showed a similar bile salt independent bile flow (y-intercept) for both groups (1.8 ± 0.7 vs. 3.2 ± 0.6 ml.min^{-1}.100 gram BW^{-1}, respectively; NS). In contrast, the bile salt-dependent flow (slope) was significantly higher in *Cftr*$^{-/-}$ mice compared to controls (20 ± 3 vs. 8 ± 3 μL/μmol, respectively; $P < 0.05$) (Fig. 1D).

3.3. Biliary bile salt profile

In their bile, *Cftr*$^{-/-}$ mice had significantly lower proportions of CA (40.3 ± 8.5 vs. 53.0 ± 7.5%; $p < 0.05$), DCA (1.6 ± 2.0 vs. 5.3 ± 2.7%; $p < 0.05$) and ω-MCA (0.9 ± 0.5 vs. 6.1 ± 2.6%; $P < 0.05$) compared to controls (Fig. 2A). The proportion of biliary primary bile salts (CA, CDCA, α- and β-MCA) was significantly decreased in *Cftr*$^{-/-}$ mice compared to controls (70% vs. 84% respectively; $p < 0.05$, Fig. 2B). Additional gas chromatographic analysis of bile showed an unexpected, ~7-fold higher content of the secondary bile salt UCA in *Cftr*$^{-/-}$ mice compared to controls (24.6 vs. 3.3% respectively; $P < 0.01$, Fig. 2A). Mass spectrometry confirmed the identification and abundance of UCA in CF mice. As a result of the increased proportion of the hydrophilic UCA, the biliary bile salt hydrophobicity index of *Cftr*$^{-/-}$ mice was significantly lower compared to controls (-0.25 ± 0.09 vs. -0.13 ± 0.05 respectively; $P < 0.05$; Fig. 2C). We tested whether the severity of liver pathology correlated with our current bile acid data but did not find a significant correlation.

3.4. Biliary lipids profile

There was no significant difference between the biliary phospholipid concentration of *Cftr*$^{-/-}$ and control mice (Fig. 3A).

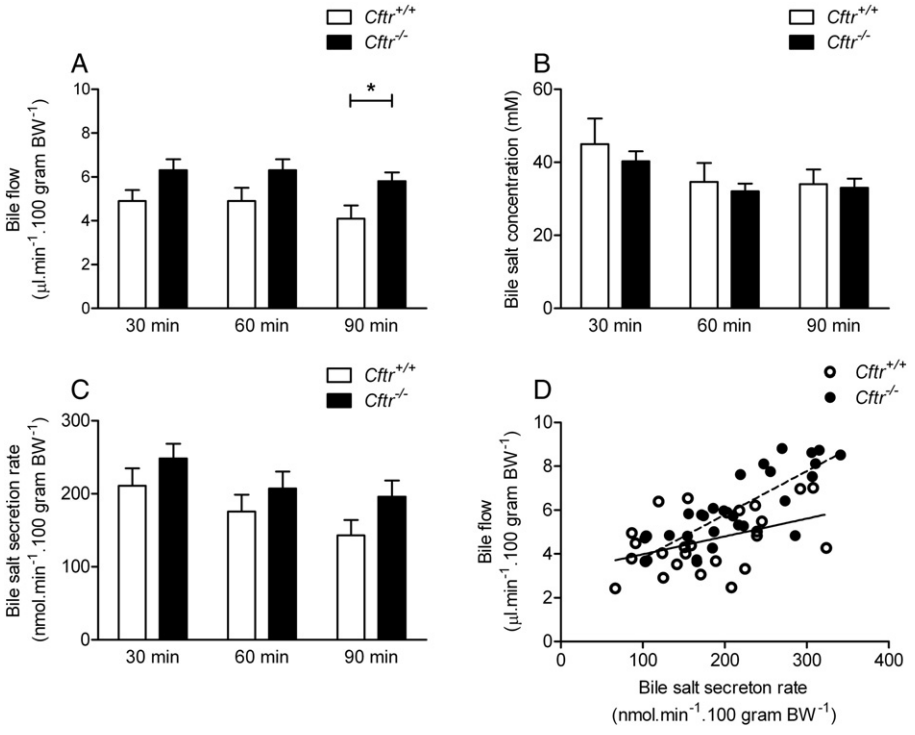

Fig. 1. **Bile production and bile salt secretion parameters of *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice**. Biliary bile flow (A), bile salt concentration (B) bile salt secretion rate (C) and bile salt secretion rate (BSSR) vs. bile flow in Cftr knockout mice (*Cftr*$^{-/-}$) and control littermates (*Cftr*$^{+/+}$). Panel D shows the correlation between all individual BSSR vs. bile flow data within both groups. Here there is a significant correlation between BSSR and bile flow in *Cftr*$^{+/+}$ (Spearman's Rho 0.394, $P = 0.042$), as well as in *Cftr*$^{-/-}$ mice (Spearman's Rho = 0.762, $P = 0.000$). Data are presented as means ± SEM of n = 9 mice per group. *P-value < 0.05.

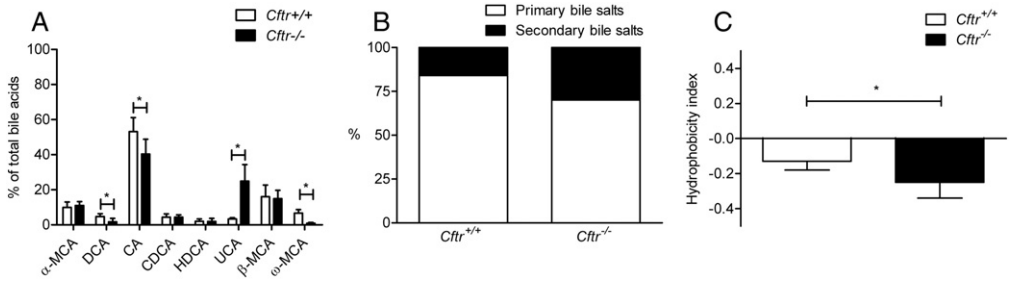

Fig. 2. **Biliary bile salt profile of *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice**. Biliary bile salt profiles (A). Abbreviations: CA = cholic acid, DCA = deoxycholic acid, CDCA = chenodeoxycholic acid, HDCA = hyodeoxycholic acid, UDCA = ursodeoxycholic acid, UCA = ursocholic acid, MCA = muricholic acid. Data are presented as a percentage of different bile salt species (n = 6–7 mice per group). Relative distribution between primary bile salt content and secondary bile salt (B). Primary bile salt content is decreased in *Cftr*$^{-/-}$ mice, whereas secondary bile salt content is increased (P = 0.004). Heuman index (C) of the total biliary bile salts representing the hydrophobicity of bile salts.

In both *Cftr*$^{-/-}$ and controls, the phospholipid secretion rate was linearly related to the bile salt secretion rate, despite the significant differences in biliary bile salt hydrophobicity (Fig. 3B). We found no difference in the biliary phospholipid to bile salt ratio between *Cftr*$^{-/-}$ and control mice (Fig. 3C), nor in biliary cholesterol concentration or cholesterol secretion rate (data not shown).

3.5. Fecal bile salt composition

The fecal bile salt loss of *Cftr*$^{-/-}$ mice was ~80% higher compared to controls (Fig. 4A). Mass spectrometry analysis showed that, in correspondence with the biliary bile salt profile, *Cftr*$^{-/-}$ mice also had a profoundly higher fecal UCA content compared to controls (27 vs. 1%, respectively; P < 0.01, Fig. 4B). In feces, *Cftr*$^{-/-}$ mice had a significantly lower DCA (11.4 ± 10.3 vs. 42.0 ± 10.7% respectively; P < 0.01), HDCA (0.3 ± 0.2 vs. 2.2 ± 0.9% respectively; P < 0.01) and ω-MCA (1.2 ± 0.9 vs. 18.1 ± 8.3 respectively; P < 0.01) content compared to controls. On the other hand, *Cftr*$^{-/-}$ mice had a significantly higher fecal CA content compared to controls (26.5 ± 9.1% vs. 8.4 ± 7.4% respectively; P < 0.01).

To address whether the observed differences in bile salt composition were genotype or environment (microflora) related, we performed fecal bile salt analysis in mice of C57BL/6 J *Cftr*$^{-/-}$ mice and controls from a different colony at another institution (courtesy of Dr. R.C. De Lisle, University of Kansas, Kansas City, Missouri; N = 5 per group). We found that the bile salt composition, in particular the presence of UCA in CF mice, was largely identical (data not shown).

3.6. Bacterial composition of fecal flora

We found no difference in the total fecal bacterial DNA content between *Cftr*$^{-/-}$ and controls. We found a significant decrease of in the fecal Bacteroides content in *Cftr*$^{-/-}$ mice compared to controls $(1.1 \pm 0.4).10^8$ vs. $(1.6 \pm 0.3).10^7$ bacteria/gram feces respectively; P < 0.01, Fig. 5A). No differences were observed in fecal Clostridium or Lactobacilli content between *Cftr*$^{-/-}$ mice and controls (Figs. 5B and 5C).

4. Discussion

We hypothesized that the C57BL/6 J CF *Cftr*$^{-/-tm1Unc}$ mice develops hepatic histopathology in the context of a hydrophobic and cytotoxic biliary bile salt profile. In contrast to our assumptions, we actually found that the bile of *Cftr*$^{-/-tm1Unc}$ mice was more hydrophilic and that the bile production in *Cftr*$^{-/-tm1Unc}$ mice, was in fact higher than in control mice. We assume that the higher bile production is related to the increased

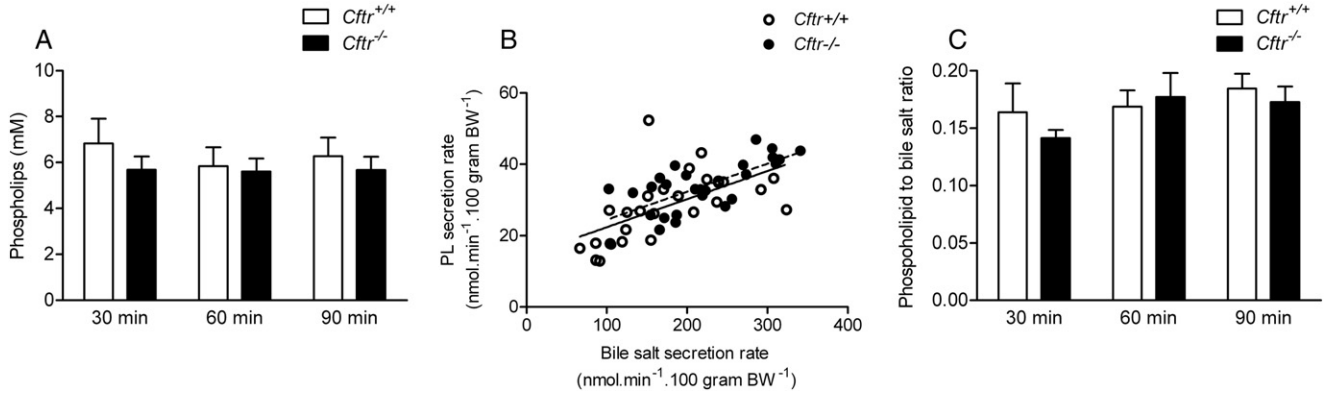

Fig. 3. **Biliary phospholipid of *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice**. Phospholipid concentration (A) the phospholipid secretion vs. bile salt secretion rate (B) and phospholipid to bile salt ratio (C) in *Cftr* knockout mice (*Cftr*$^{-/-}$) and control littermates (*Cftr*$^{+/+}$), n = 9 mice per group. If appropriate data presented as means ± SEM of n = 9 mice per group.

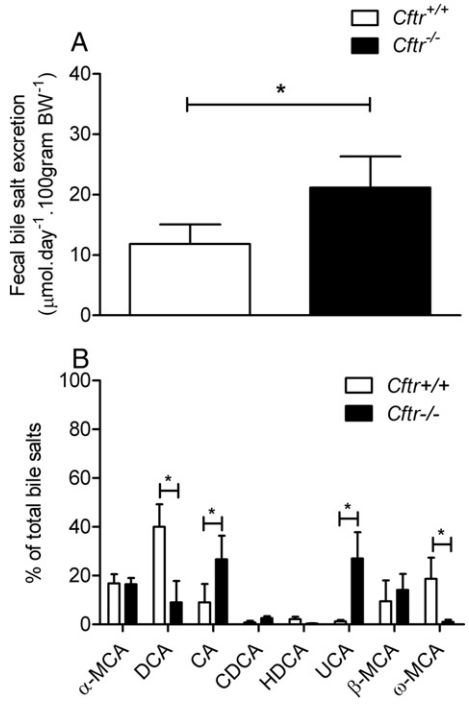

Fig. 4. **Fecal bile salt excretion and fecal bile salt profile of *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice**. Total bile salt excretion (A), presented as means ± SEM. Bile salt profiles (B), data are presented as a percentage of different bile salt species present in bile. Abbreviations: CA = cholic acid, DCA = deoxycholic acid, CDCA = chenodeoxycholic acid, HDCA = hyodeoxycholic acid, UDCA = ursodeoxycholic acid, UCA = ursocholic acid, MCA = muricholic acid. n = 9 mice per group. *P-value < 0.05.

biliary enrichment with hydrophilic bile salts, including UCA (15). This conclusion is supported by the significantly higher bile salt-dependent bile flow, found in the *Cftr*$^{-/-}$ mice.

Other mechanisms for the development of liver pathology in the *Cftr*$^{-/-tm1Unc}$ have been suggested. Blanco et al. describe that chemical induction of colitis, by dextran, aggravated bile duct injury in *Cftr*$^{-/-tm1Unc}$ mice compared to controls (16). In a in a follow-up study they describe a decreased peroxisome proliferator-activated receptor alpha expression in *Cftr*$^{-/-tm1Unc}$ (17). Different from our bile salt hydrophobicity hypothesis, their findings point more towards a Cftr related, immunologic/inflammatory genesis of CFLD.

The increased UCA enrichment in bile and feces of *Cftr*$^{-/-}$ mice is an unexpected and notable finding. UCA is formed, in the intestine, by the bacterial transformation of CA. In normal conditions, UCA is rapidly transformed to DCA, by the bacterial microflora. As a result the contribution of UCA to the bile salt pool, is usually very small. The high UCA enrichment of bile and feces found in the *Cftr*$^{-/-tm1Unc}$ mice is, therefore, the related to, Cftr dependent, alteration in the bacterial microflora normally involved in the transformation of CA to DCA.

Elaborating on that subject, we indeed observed significant differences in the intestinal bacterial composition between *Cftr*$^{-/-}$ and control mice. In particular we demonstrated that the contribution of Bacteroides bacteria was significantly lower in feces of *Cftr*$^{-/-}$ mice. Bile salt deconjugation activity is found in many bacterial species including Bacteroides. As a result of this observation, difference in Bacteroides content, could theoretically, be related to the observed variation in biliary and fecal bile salt composition. However, we realize that our results, on fecal bacterial composition, do not prove causality in this matter.

We found comparable differences in bile salt composition between *Cftr*$^{-/-tm1Unc}$ and control mice, housed in a different animal facility. These results suggest a reasonable reproducibility of our findings. Nevertheless, we realize, the Cftr/bile salt/microflora interaction is probably not a genotype related, fixed condition. The latter is illustrated by our previous study with C57BL/6 J CF mice (18). In this study we did not see any fecal UCA, despite the fact that the mice were bred and fed under the same conditions.

Our current results, in the *Cftr*$^{-/-tm1Unc}$ mice model, differ in several aspects from the results found in human studies on bile salt metabolism and composition, in CF. Strandvik et al. described an increase of the primary bile acids in urine and serum of CF patients. They reported a correlation between the serum bile salt composition and the extent of liver disease in cystic fibrosis. However, different from our current findings in mice, they do describe a predominance of primary bile salt species. They suggest their findings to be related to specific changes of intestinal factors in CF conditions. Smith et al. described the duodenal, urinary and serum bile salt compositions of CF patients with and without CFLD (19). They report a reduction of secondary bile salts in CF patients compared to healthy controls. Based on their results, these researchers propose a role of secondary, more hepatotoxic, bile salts in the pathogenesis of CFLD.

UCA is a naturaly occurring, highly choleretic bile salt. In our model, the enrichment of UCA, leads to a significantly increased bile salt-dependent flow and to a low biliary hydrophobicity in

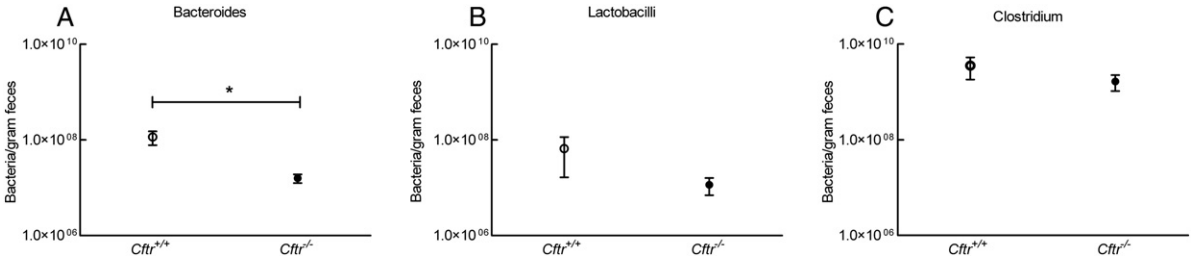

Fig. 5. **Fecal bacterial content of *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice.** Fecal content (bacteria per gram feces) of Bacteroides (A) Lactobacillus (B) and Clostridium coccoides (C) based on microbial DNA measurement. Data are presented as means ± SEM of n = 6–9 mice per group. *P-value < 0.05.

Cftr$^{-/-}$ mice. UCA supplementation has been reported to decrease biliary excretion of phospholipid and cholesterol in rats (20). However, in our current study we did not see UCA effects on the biliary phospolipid and cholesterol excretion. It is notable that, in this study, we did find any ursodeoxycholic acid (UDCA) in either bile or stools. Different from our current results, we did see low amounts of UDCA (<5% of total) in another Cftr knockout mouse model (Cftr$^{-/-tm1CAM}$) (21). The presence of UDCA in CF conditions may, therefore, be strain-specific. Additionally, in humans, Smith et al. reported significantly greater proportions of endogenous ursodeoxycholic acid in bile of CF patients without liver disease when compared to CF subjects with liver disease (19). Their results imply a possible protective role, of endogenous UDCA, in the development of CFLD.

The fact that, the mice in this study were exclusively fed a liquid diet (Peptamen) may have influenced bile salt composition results. The *Cftr*$^{-/-tm1Unc}$ mice remain dependent on liquid feeding, to prevent lethal intestinal obstruction (22). *Cftr*$^{-/-}$ mice and controls were both fed the liquid diet. Therefore, in itself, the liquid diet cannot directly explain the differences in bile salt composition between *Cftr*$^{-/-}$ mice and controls. On the other hand, it cannot be excluded that there are differences in the way, Cftr$^{-/-}$ and control mice interact with the liquid diet, for example, on the level of the intestinal bacterial microflora. In this manner, theoretically, a liquid diet could, indirectly, result in differences in bile salt composition.

We found an increased fecal bile salt excretion in *Cftr*$^{-/-tm1Unc}$. Fecal bile salt loss is an intrinsic and consistent phenotype of human CF patients as well as of experimental CF animal models (23,24). In a previous study, we saw that intestinal bile salt loss in *Cftr*$^{-/-}$ mice is compensated by an increased hepatic bile salt synthesis (21). It has been suggested that the underlying mechanism of increased fecal bile salt excretion involves increased fecal fat excretion in CF conditions (25). However, previously we reported that increased fecal bile salt loss was also observed in CF mice without fat malabsorption (26). Theoretically, an increased transit time could be related to the increased fecal bile salt output. However, CF mice have formed, solid, stool and do not present with diarrhea. Additionally we did not find any indication for an altered intestinal transit time of dietary lipids in ΔF508-CF mice, compared to wild type mice, despite a similarly, increased, fecal bile salt excretion in ΔF508-CF mice as in *Cftr*$^{-/-tm1Unc}$ mice (24).

In our current study we found that the hepato-biliary pathology of *Cftr*$^{-/-tm1Unc}$ mice is not related to increased biliary bile salt hydrophobicity or cytotoxicity. However, *Cftr*$^{-/-tm1Unc}$ mice do display distinctive changes in the metabolism and enterohepatic circulation of the bile salts. Recent discoveries in the intestinal regulation of bile salt synthesis shed a new light on the importance and role of the intestinal bile salt composition. The reported differences in bile salt metabolism indicate a Cftr-dependent influence on intestinal bacterial microflora and microbial bile salt metabolism. These conclusions are supported by the fact that our findings coexisted with a significant smaller quantity of fecal Bacteroides bacteria. It is tempting to speculate that these processes play a role in the development of the CF intestinal and hepatic phenotype.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2014.12.010.

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